RESEARCH PAPER

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Developmental stage-and concentration- specific sodium nitroprusside application results in nitrate reductase regulation and the modification of nitrate metabolism in leaves of *Medicago truncatula* plants

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Abbreviations: NO, nitric oxide; SNP, sodium nitroprusside; ROS, reactive oxygen species; RNS, reactive nitrogen species; NR, nitrate reductase

Nitric oxide (NO) is a bioactive molecule involved in numerous biological events that has been reported to display both pro-oxidant and antioxidant properties in plants. Several reports exist which demonstrate the protective action of sodium nitroprusside (SNP), a widely used NO donor, which acts as a signal molecule in plants responsible for the expression regulation of many antioxidant enzymes. This study attempts to provide a novel insight into the effect of application of low (100 μM) and high (2.5 mM) concentrations of SNP on the nitrosative status and nitrate metabolism of mature (40 d) and senescing (65 d) *Medicago truncatula* plants. Higher concentrations of SNP resulted in increased NO content, cellular damage levels and reactive oxygen species (ROS) concentration, further induced in older tissues. Senescing *M. truncatula* plants demonstrated greater sensitivity to SNP-induced oxidative and nitrosative damage, suggesting a developmental stage-dependent suppression in the plant's capacity to cope with free oxygen and nitrogen radicals. In addition, measurements of the activity of nitrate reductase (NR), a key enzyme involved in the generation of NO in plants, indicated a differential regulation in a dose and time-dependent manner. Furthermore, expression levels of NO-responsive genes (*NR, nitrate/nitrite transporters*) involved in nitrogen assimilation and NO production revealed significant induction of *NR* and *nitrate transporter* during long-term 2.5 mM SNP application in mature plants and overall gene suppression in senescing plants, supporting the differential nitrosative response of *M. truncatula* plants treated with different concentrations of SNP.

Introduction

Over the past years the free radical nitric oxide (NO) has emerged as a signal molecule in many important physiological processes in higher plants in a similar fashion to reactive oxygen species (ROS).[1] It is also apparent that NO, along with ROS, is involved in plants' responses to a multitude of environmental stimuli such as salinity, drought, high light intensity and mechanical wounding.[2,3] Interestingly, the existence of a cross-talk between ROS and NO is well documented and has been recently reviewed.[4] Several excellent reviews exist which provide latest insights into this multifaceted molecule (see refs. 5 and 6). NO is biologically active at a concentration of 1 nmol/l and participates in signaling cascades that drive plant growth and developmental processes.[7] Current knowledge of the role of NO in plants is still rather limited by the relative lack of mutants with altered NO production as well as molecules that can sense and transduce NO signals.[8]

NO donors are compounds that produce NO when applied to biological systems and are able to either mimic an endogenous NO-related response or substitute for an endogenous NO

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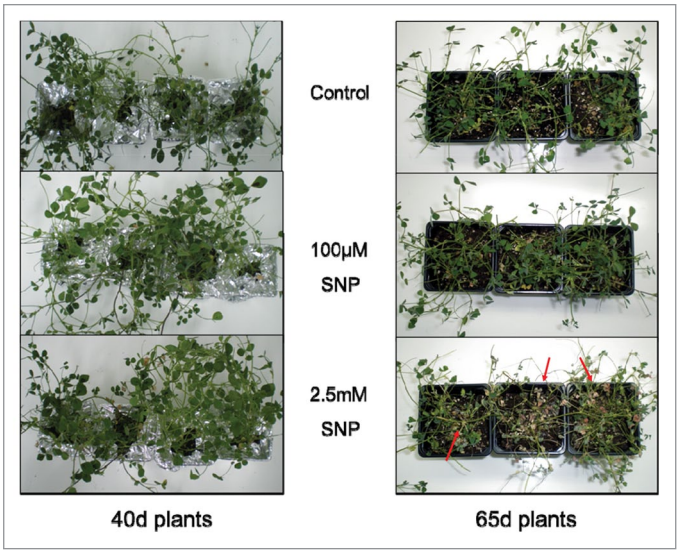


Figure 1. Phenotypic response of *Medicago truncatula* plants five days after application of varying SNP concentrations by vacuum infiltration. Senescing (65 d) plants treated with 2.5 mM SNP show increased damage levels indicated by wilted, chlorotic leaves.

deficiency.[9] The most commonly used one is sodium nitroprusside (SNP), an NO^+ donor.[10] According to Zandonadi et al.,[11] the reason SNP is so widely used is due to its relatively low cost and well-documented application as NO donor, while Floryszak-Wieczorek et al.[9] state that SNP gives a continuous, long-lasting NO production compared with other NO donors which is often desirable.

The term reactive nitrogen species (RNS) has been formulated to designate NO and the NO-derived molecules such as nitrogen dioxide ($^{\cdot}NO_2$), peroxynitrite ($ONOO^-$), S-nitrosothiols (RSNOs) and S-nitrosoglutathione (GSNO).[12] Nitrosative stress is induced by pathophysiological levels of NO and S-nitrosothiols, resulting from the nitrosylation of critical protein cysteine (Cys) thiols (S-nitrosylation) and metal co-factors.[13] Although NO is characterized by its inherent toxic nature and is known to potentially be damaging to cells depending on its concentration and on the situation,[14] the NO-triggered defense responses are now widely recognized.[15] NO can also have a direct, protective effect against abiotic stress factors, as it alleviates the deleterious effects of ROS in establishing stress tolerance responses,[15] partly by increasing the activity of antioxidant enzymes.[16]

Nitrate reductase (NR) is the first enzyme in the nitrate assimilation pathway, reducing nitrate into nitrite.[17] Cytosolic NR is also rapidly emerging as one of the main sources of NO in plants under aerobic conditions.[18] Plants have several mechanisms to regulate the activity, level and location of NR in response to a range of environmental conditions and chemicals.[19] The signals that alter NR activity are tightly regulated at the transcriptional and post-translational levels.[20,21] In higher plants, NR is rapidly inactivated/activated by phosphorylation/dephosphorylation, respectively, in response to different environmental stimuli and treatments.[22]

Early studies on nitrate signaling demonstrated that nitrate induces the de novo synthesis of NR.[23] Subsequent work also demonstrated that nitrate induces other genes in the nitrate assimilation pathway, namely nitrate transporters (*NRT*s).[24] The uptake of nitrate (NO_3^-) by plant cells relies on transport systems, usually membrane carriers belonging to either NRT1 or NRT2 transporter families (see ref. 25) or a channel into the vacuole of which the expression has been shown to be enhanced by nitrate.[26] In addition to the nitrate uptake system, plants have an inducible nitrate efflux system, requiring both RNA and protein synthesis; however, it has a much slower turnover rate than the uptake system.[27] Considering that the physiological concentration of nitrite in cells can be very low (μM range),[28] a nitrite transporter might also be of importance in nitrite uptake by the inner envelope of higher plant chloroplasts, in order to prevent the accumulation of toxic nitrite in the cytosol.[29]

The present study attempts to elucidate the importance of different concentrations of SNP and the NO induced in cellular integrity and ROS/RNS interplay, as well as in NR expression and regulatory activity by investigating the dose-, developmental stage- and time-dependent effect of SNP application in NR activity. It also examines the role of SNP and the NO produced as a signal regulating *NR* gene expression and triggering the mobilization of other genes implicated in N metabolism and transport (i.e., nitrate/nitrite transporters), thus providing novel insights into the complex regulation of NO metabolism and the cross-talk between ROS and RNS in *M. truncatula* plants.

Results

Physiological characterization of SNP-treated *M. truncatula* plants. Vacuum infiltration of *M. truncatula* plants with different concentrations of SNP and subsequent macroscopic observation five days after SNP application revealed no phenotypic differences in 40-d-old plants, whereas 65-d-old plants treated with 2.5 mM SNP showed increased damage levels indicated by wilted, chlorotic leaves in comparison with control and 100 μM SNP-infiltrated samples (**Fig. 1**).

Effect of SNP in photosynthetic pigment content. Similar trends were observed in carotenoid and total chlorophyll content: 2.5 mM SNP application resulted in significant reduction in both photosynthetic pigment contents of senescing plants, with a more dramatic effect after 24 h of SNP application (**Fig. 2**). Carotenoid and total chlorophyll content was also reduced in 40-d-old plants, although the differences were not statistically significant.

Cellular damage levels and ROS measurements. A temporal examination of cellular damage levels was performed by means of spectrophotometric determination of lipid peroxidation, a commonly used marker of oxidative damage. Increasing SNP concentrations resulted in increasing membrane damage, reaching maximum levels at 24 h after application in both mature and senescing plants. The latter demonstrated much higher lipid peroxidation levels in response to SNP application, while control (0 d plants) showed higher constitutive lipid peroxidation (**Fig. 3A**). *In situ* histochemical localization of lipid peroxidation with the use of Schiff's reagent demonstrated similar overall patterns (**Fig. 3B**).

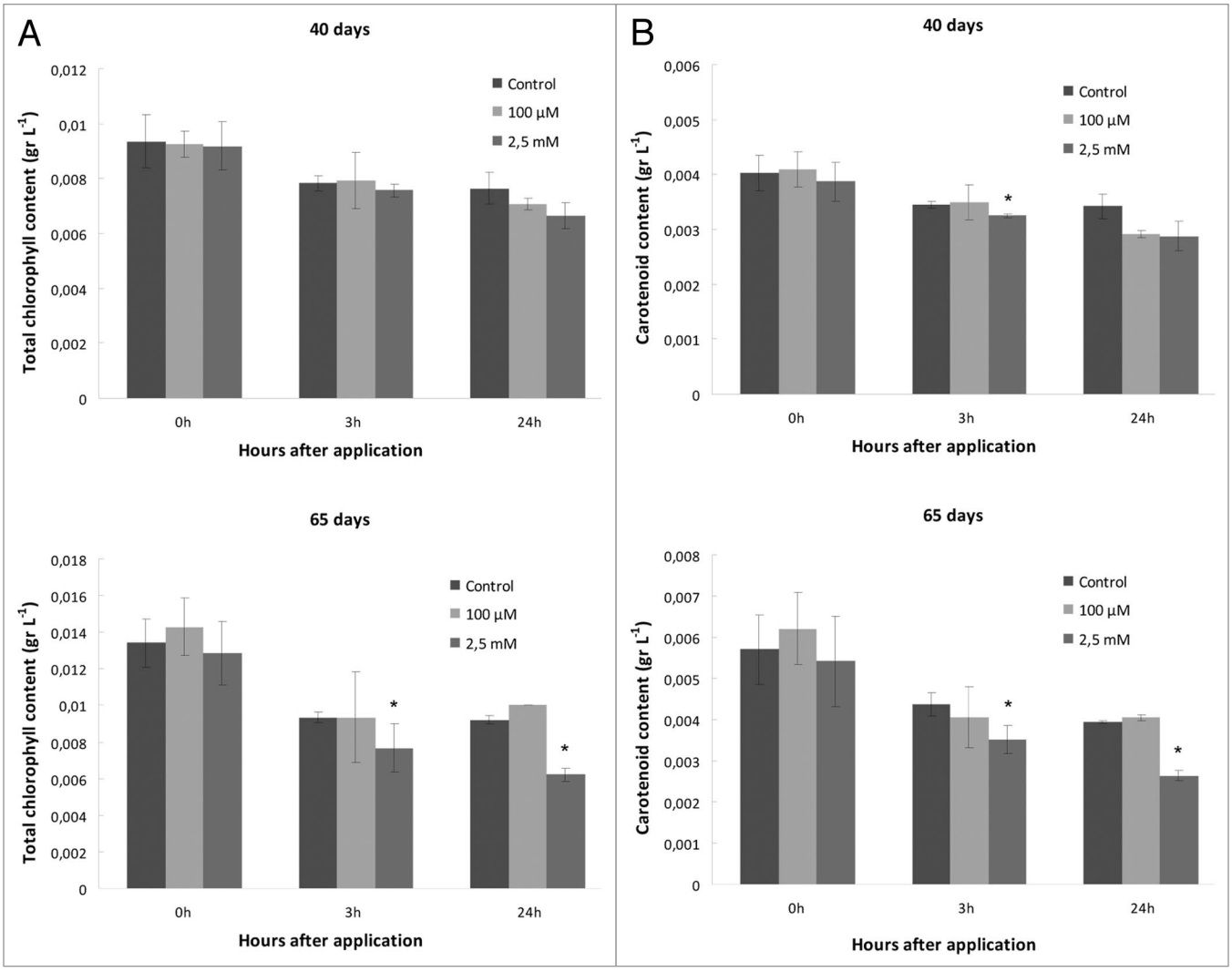


Figure 2. Effect of SNP application on photosynthetic pigment content. (**A**) Total chlorophyll (α + β) content, (**B**) Carotenoid content (where upper rows represent measurements made with 40 d plants, while lower rows indicate measurements made with 65 d plants). Asterisks denote statistically different values according to the Tukey pairwise comparison test ($p < 0.05$). Values are means ± SE (n = 3).

Spectrophotometric determination of cellular reactive oxygen (H_2O_2) species content in SNP-treated plants showed a gradual increase in H_2O_2 in both mature and senescing plants, reaching maximal levels at 24 h after 2.5 mM SNP application (**Fig. 4A**). Overall, higher concentrations of SNP resulted in increased H_2O_2 concentration, further increasing in older tissues. Hydrogen peroxide content was also increased after 3 h, although the increase was not statistically significant. *In situ* histochemical localization of hydrogen peroxide with the use of DAB demonstrated similar overall patterns (**Fig. 4B**).

Effects of NO content on NR enzymatic activity. Nitrite-derived nitric oxide content in SNP-treated leaves was measured using the Griess reagent (see "Materials and Methods" section). Similar overall trends were observed in both developmental stages of *M. truncatula* plants, demonstrating increasing nitrite-derived NO content with increasing SNP concentrations applied. Maximum NO contents were recorded in both mature and senescing plants infiltrated with 2.5 mM SNP after 24 h (**Fig. 5A**).

To further elucidate the possible role and mechanism by which SNP regulates NR activity in *M. truncatula* plants, NR enzymatic activity was measured in mature and senescing plants after low (100 μM) and high (2.5 mM) SNP application. At the lower SNP application (100 μM), low SNP concentration caused a short-term (3 h) activation of NR activity further increased after the long-term effect of SNP application (24 h) in mature and to a further extent in senescing plants (**Fig. 5B**). In addition, the short-term application of the higher SNP concentration (3 h, 2.5 mM SNP), also caused an increase in NR activity (**Fig. 5B**).

Although the stimulating effect of NO in NR activity was observed at the lower SNP concentration application, higher concentrations partly inhibited NR activity, as indicated by the lower levels of NR activity after 24 h of 2.5 mM SNP application. This effect was more obvious in senescing plants (65 d) treated with the higher SNP concentration (**Fig. 5B**).

Expression analysis of genes implicated in NO metabolism after SNP application. The expression patterns of three key

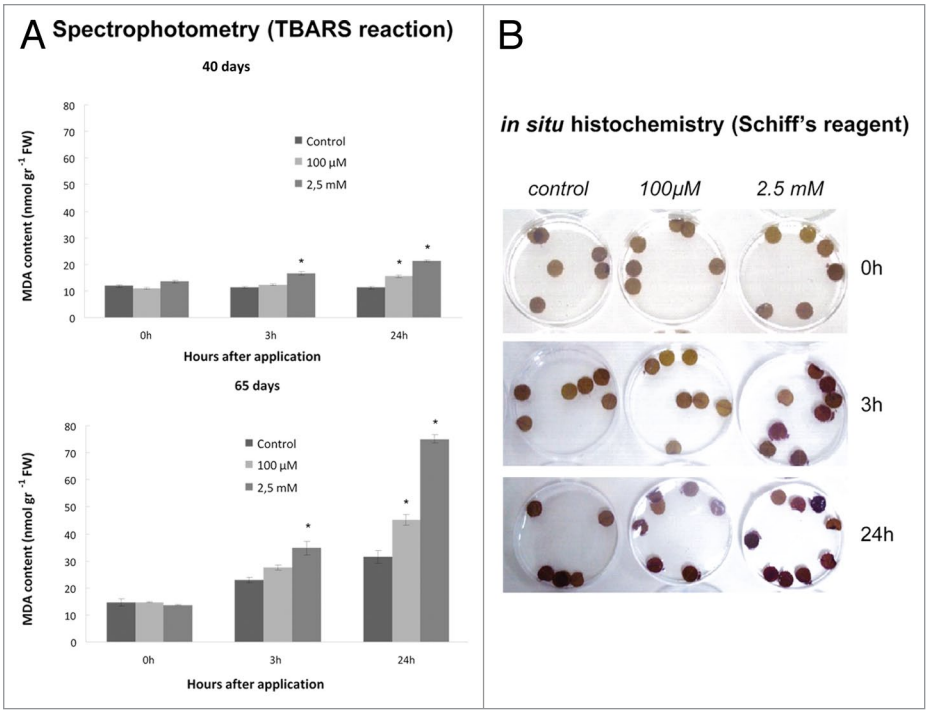


Figure 3. Effect of SNP application on cellular damage as indicated by lipid peroxidation (MDA content). (**A**) quantitative analysis by spectrophotometry, where upper row represents measurements made with 40 d plants, while lower row indicates measurements made with 65 d plants. (**B**) qualitative analysis by in situ histochemistry using Schiff's reagent. Figure presents 40 d old plants; similar trend was observed with 65 d plants (data not shown). Asterisks denote statistically different values according to the Tukey pairwise comparison test ($p < 0.05$). Values are means ± SE ($n = 3$).

genes (*nitrate and nitrite transporters, nitrate reductase*) involved in the NO metabolism was assayed by a quantitative real-time RT-PCR approach, revealing differential regulation in leaves of both mature and senescing plants for the genes examined (**Fig. 6**). Analysis of the expression of genes encoding proteins associated with NO biosynthesis (*NR*) or transporters (*nitrate/nitrite transporter*) was performed following 3 h and 24 h of 100 μM and 2.5 mM SNP application in mature and senescing plants (**Fig. 6**).

A similar trend of suppressed expression of *NR* as well as *nitrite* and *nitrate transporter* genes was observed in senescing (65 d) plants. Contrarily, a significant induction was observed in *NR* and *nitrate transporter* gene expression during the long-term effect (24 h) of the higher SNP concentration (2.5 mM) in 40-d-old plants (**Fig. 6**). Thus, there was a significant induction in transcript abundance of *NR* and to a lesser extent in *nitrate transporter* in NO-accumulating cells. By contrast, an overall suppression of the *nitrite transporter* gene was observed in mature and to a further extent in senescing plants (**Fig. 6**).

Discussion

Nitric oxide is a key player in several biological cellular processes, acting either as a signaling or as a toxic molecule in plants.[1,30] It is also acknowledged as a major component in the establishment of plant symbiosis with nitrogen-fixing bacteria.[31] The present study was performed in an attempt to elucidate the effect of application of two different concentrations of SNP, a widely used NO donor, in *M. truncatula* plants in relation with RNS/ROS signals and cellular NO metabolism. Among the different types of NO donors, SNP was used because of its high efficiency to release NO in plant cells,[32] as well as continuous, long-lasting NO production.[9] Such a focus was also largely given due to the importance and frequency of SNP use in Plant NO studies (> 600 research articles published during the past 10 years; Source: http://www.scopus.com).

In order to characterize the effect of SNP and NO produced in *M. truncatula* plants in a concentration-dependent manner, two different SNP concentrations (100 μM and 2.5 mM) were chosen, based on previous studies.[33,34] Moreover, the important role of NO during senescence (see Leshem et al.[35]) prompted us to investigate the effect of SNP application in *Medicago truncatula* plants of different developmental stages (40-d-old mature plants and 65-d-old senescing plants), further supported by the investigation of short-term (3 h) and long-term (24 h) SNP application effect.

Carotenoids are low molecular weight compounds that act as non-enzymatic antioxidants primarily quenching singlet oxygen,[36] while chlorophyll degradation is a known stress-related marker.[37] Phenotypic plant observations in 2.5 mM SNP-treated plants revealed increased visible damage levels and a decline in Car and Chl contents in senescing plants (**Fig. 2**), while treatment with 100 μM SNP did not affect the plant's growth and viability (**Fig. 1**) and the cellular status of the cell. This result is in accordance with Sung and Hong,[38] indicating the effect of different NO concentrations in plant development and a similar decline in Car and Chl,[37,39] further suggesting that Chl catabolism is highly regulated during development and senescence.[39,40] One possible explanation for the observed phenotypic and physiological behavior of the SNP-treated *M. truncatula* plants may be due to the major induction of ROS/RNS production, according to the increased sensitivity of various plants to oxidative damage under severe stress conditions.[30,41] Indeed, higher NO concentration (nitrosative stress) resulted in H_2O_2 accumulation, accompanied by increased cellular damage levels (accumulation of MDA) in mature plants, further induced in senescing plants (**Figs. 3 and 4**), thereby indicating its toxic role.[42] Contrarily, plants treated with the lower NO concentration resulted in lower H_2O_2 and MDA content in leaves (**Figs. 3 and 4**), indicating the protective role of NO by reacting with ROS and consequently inhibiting the detrimental effects of membrane lipid peroxidation.[43]

Interestingly, NO content is markedly increased in leaves of 2.5 mM SNP-treated mature plants compared with control

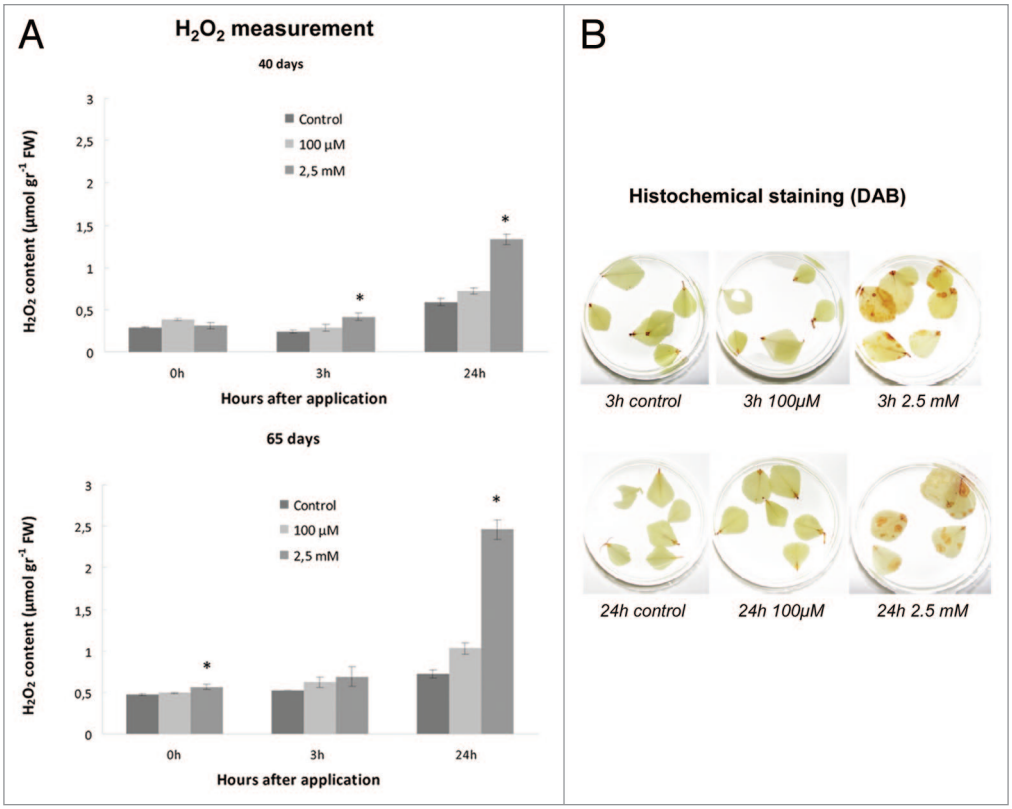


Figure 4. Effect of SNP application on hydrogen peroxide levels. (**A**) quantitative analysis by spectrophotometry, where upper row represents measurements made with 40 d plants, while lower row indicates measurements made with 65 d plants. (**B**) qualitative analysis by in situ histochemistry using DAB reagent. Figure presents 65 d old plants; similar trend was observed with 40 d plants (data not shown). Asterisks denote statistically different values according to the Tukey pairwise comparison test ($p < 0.05$). Values are means ± SE ($n = 3$).

samples after short-term (3 h) application, unlike H_2O_2 content which remains at similar levels and is only increased after long-term 2.5 mM SNP application, therefore suggesting that NO induction in leaves appears to precede H_2O_2 induction (**Figs. 4A and 5A**). NO has been shown to be involved in the signaling pathway upstream of H_2O_2 synthesis, thus justifying the observed timing of induction of the two signaling molecules.[44]

Although NO synthesis in plants is still a matter of debate,[45] NO_3^- may be reduced to NO in *M. truncatula* plants in a two-step mechanism involving NR and other systems implicated in electron transport chain.[46] In an attempt to further clarify the actual mechanism of NR regulation by NO in SNP-treated plants, it was observed that NO accumulation in *M. truncatula* plants (24 h, 2.5 mM SNP) resulted in a decline in NR activity (**Fig. 5B**), similar to previous research findings.[46,47] This is likely to be the result of NO toxicity.[42] It is also possible that higher concentrations of NO result in a negative feed-back regulatory mechanism, thus inhibiting this NO biosynthetic enzyme. However, the significant induction rather than suppression of *NR* transcripts observed in mature plants treated with 2.5 mM SNP (**Fig. 6**), suggests that NR activity is regulated by SNP at the post-translational rather than the transcriptional level. Such possibility for post-translational modification of NR activity is further supported by the fact that low concentration of SNP (100 μM) caused a transient increase in NR activity, reaching maximal activation after 24 h (**Fig. 5B**) in both developmental stages, in contrast with transcript levels that were not induced but were actually suppressed in senescing plants and in transient response mature plants (**Fig. 6**). Several reports demonstrate the post-translational regulation of NR activity,[22,49,50] while SNP treatment has been shown to result in massive carbonylation, nitration and S-nitrosylation of citrus proteins.[41]

The lack of direct correlation between NR gene expression and enzymatic activity, revealing the complexity of the NR regulatory mechanism and the differential gene expression regulation according to the signal,[51] could also be partially attributed to the presence of two NR encoding genes in the *M. truncatula* genome (NR1: TC137636; Mtr.10604.1.S1_at, and NR2: TC130773; Mtr.42446.1.S1_at; see ref. 46), similarly to *Arabidopsis thaliana*.[52] It could be speculated that one or both of the genes is differentially regulated at different environmental/exogenous conditions (i.e., different SNP concentrations) and time points, or that both genes are regulated simultaneously by different metabolic pathways.

Following our observations dealing with the interaction of NO with oxidative events indicative of the plant's developmental status at both age and biochemical/physiological level, further experiments were performed in order to show whether NO SNP exhibits developmental stage-specific regulation of nitrate/nitrite uptake. In an attempt to examine whether the influx

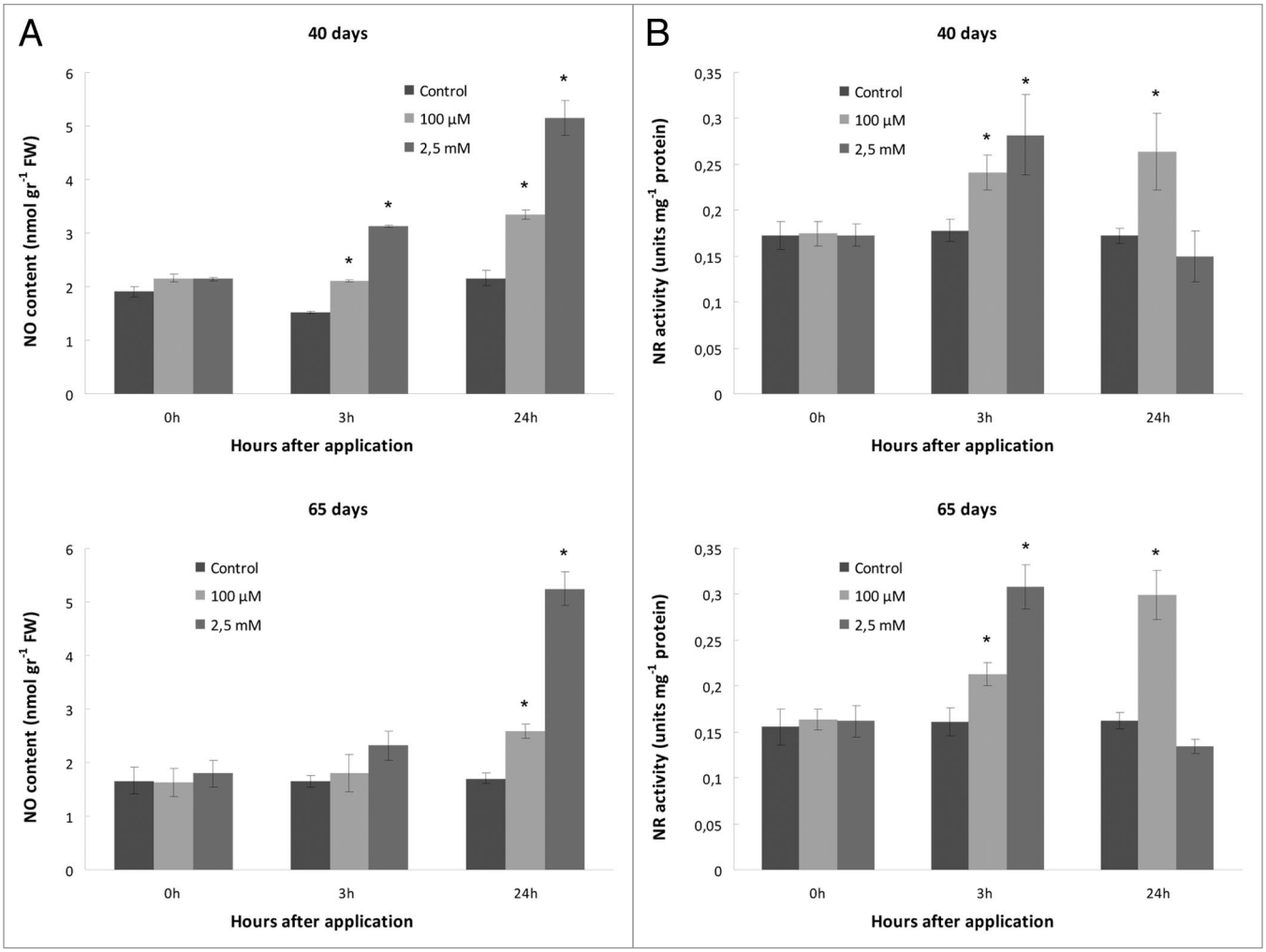


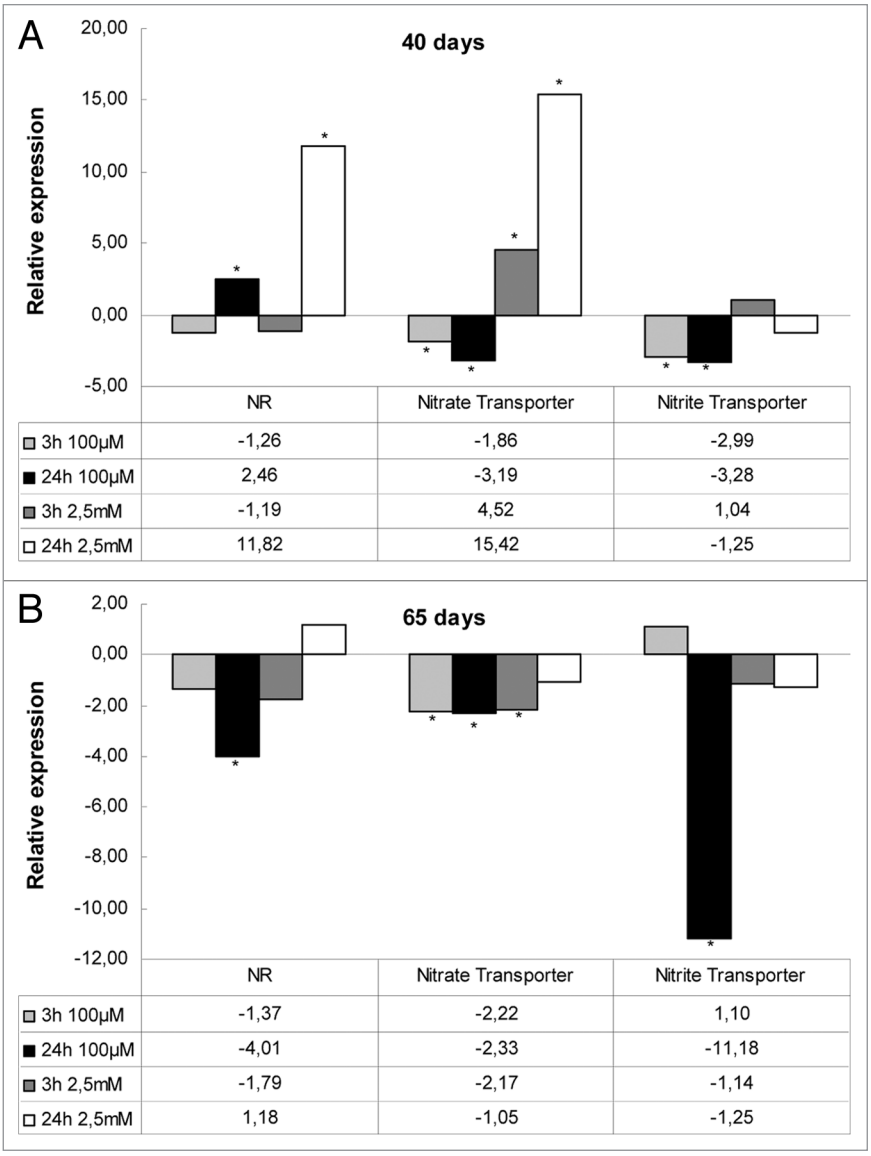
Figure 5. Effect of SNP application on nitrite-derived nitric oxide content and NR enzymatic activity. (**A**) NO measurements made with mature (40 d) and senescing (65 d) plants after lower (100 μM) and higher (2.5 mM) SNP application. (**B**) Measurements of NR enzymatic activity in mature (40 d) and senescing (65 d) plants after lower (100 μM) and higher (2.5 mM) SNP application (Upper and lower rows represent measurements made with 40 d and 65 d plants respectively). Asterisks denote statistically different values according to the Tukey pairwise comparison test ($p < 0.05$). Values are means ± SE ($n = 3$).

rather than the total pool of nitrate/nitrite is critical for the regulation of *NR* expression, *nitrate* and *nitrite* transporter gene expression analysis was performed. A gene encoding a putative nitrate transporter (UP|Q852P5 [Q852P5], Nitrate transporter, partial [28%]) was chosen based on previous microarray analyses where expression of this transporter was found to be regulated in salt-stressed *M. truncatula* plants (Filippou and Fotopoulos; data not shown), similar to observations in Arabidopsis.[53] Similarly, transcript levels of the *nitrate transporter* gene were suppressed after 3 h and 24 h of low or high SNP application in senescing plants, showing that NO assimilation may exert a repressive effect on nitrate influx.[54] Mature plants, however, demonstrated a different mode of regulation for the higher SNP concentration where the *nitrate transporter* transcript levels strongly increased in NO-accumulating cells (**Fig. 6**). It is possible however that the increased sensitivity of senescing plants to nitrosative stress leads to a negative feedback regulation of this nitrate transporter via products of NO_3^- assimilation (i.e., NO), while it is constitutively expressed in mature plants at higher NO concentrations.[55]

Focusing on mature plants displaying highest NO concentration (24 h, 2.5 mM SNP), we tried to correlate NR activity with *nitrate transporter* expression levels, since the nitrate influx rather than the total pool is critical for the regulation of NR expression and activity.[56] Furthermore, the regulatory function of NO on NR depending on levels of nitrate supply is also established.[34] Transcript abundance of the *nitrate transporter* gene in NO-accumulating cells (24 h, 2.5 mM SNP; **Figure 6**) coupled with a significant decline in NR activity (**Fig. 5**). Similarly, in an NR-deficient line, major induction of the *NRT2* mRNA transporter was observed in mature plants leading to NO_3^- accumulation.[17,55] However, NO accumulation was also shown to inhibit NR leaf activity in senescing *M. truncatula* plants (**Fig. 5**), although gene expression of the nitrate transporter strongly diminished in leaves after SNP application (**Fig. 6**), probably due to senescence-induced deregulation of the plant metabolic mechanisms.[57]

Considering the other different NO concentrations observed (3 h 2.5 mM, 3 h and 24 h 100 μM SNP), a rather complex regulation of NR expression/activity and nitrate transporter expression is observed, implying that feedback regulation may be occurring in both developmental stages of *M. truncatula* plants, not only by modifying the expression of *NR* but also possibly via transcriptional or post-translational modification of the nitrate transporter, as a result of the general regulatory role of NO on components of multiple plant metabolic pathways.[58]

Expression analysis was further elaborated by focusing on the impact of SNP treatment on expression of nitrite transporters, as the effect of nitrite content on NR activity is well established.[51] A putative nitrite transporter (T10255 nitrite transport protein, partial [29%]), showing differential regulation in *M. truncatula* salinity-stressed plants in a similar fashion to the nitrate transporter following a microarray analysis approach (Filippou and Fotopoulos; data not shown), was further analyzed in SNP-treated plants. A general trend of suppressed expression was observed in both developmental stages (**Fig. 6**), while NO accumulation resulted in a parallel suppression of *nitrite transporter* transcripts and NR activity in mature plants (**Figs. 5 and 6**), suggesting a negative feed-back regulatory mechanism for NO, a by-product of nitrite reduction, in excess amounts.[59] Plants need to adapt a defense mechanism since NO accumulation might be toxic for the cell leading to cell death,[42] thus supporting the inactivation of the nitrite transporter and NR activity observed (**Fig. 5 and 6**). Furthermore, lower NO concentration (24 h, 100 μM SNP) inside the cell appears to be critical, acting as an inducer of NR expression and activity to form more NO. As a result, *nitrite transporter* expression is suppressed, since there is no necessity to activate the transporter for nitrite influx and further NO generation.



	NR	Nitrate Transporter	Nitrite Transporter
3h 100μM	-1,26	-1,86	-2,99
24h 100μM	2,46	-3,19	-3,28
3h 2,5mM	-1,19	4,52	1,04
24h 2,5mM	11,82	15,42	-1,25

	NR	Nitrate Transporter	Nitrite Transporter
3h 100μM	-1,37	-2,22	1,10
24h 100μM	-4,01	-2,33	-11,18
3h 2,5mM	-1,79	-2,17	-1,14
24h 2,5mM	1,18	-1,05	-1,25

Figure 6. NO effect on gene expression profiles of NO-responsive genes (*NR* and *nitrate/nitrite transporters*). Gene expression analysis was determined by qRT-PCR in leaves of *Medicago truncatula* Jemalong A17 plants vacuum-infiltrated with 100 μM and 2.5 mM SNP at 3/24 h in mature (40 d (**A**) and senescing (65 d (**B**) plants. Asterisks denote statistically different values according to pairwise fixed reallocation randomization test ($p < 0.05$) ($n = 3$).

Overall, the results presented in the current study support the notion that SNP and the NO produced can act as either a protective or cytotoxic molecule in a concentration-dependent manner. Senescing *M. truncatula* plants demonstrated greater sensitivity to NO-induced oxidative and nitrosative damage, as well as repression of both NO_3^- uptake and assimilatory systems by NO_2^- and NR activity, suggesting a developmental stage-dependent suppression in the plant's capacity to cope with free oxygen and nitrogen radicals. In addition, our findings underscore an important cross-talk between H_2O_2 and NO signaling pathways in response to nitrosative stress. However, it should be noted that certain responses could also be potentially attributed, at least partially, to the effect of side products produced by SNP such as cyanide,[59,60] although other studies reported no CN^- effect using SNP to trigger NO-induced processes.[16] Furthermore, the genomic complexity of *NR* and other NO-responsive genes regulated by SNP and the different expression patterns observed pose the necessity for full transcriptomic analyses that would provide more conclusive evidence. In closing, fully understanding and deciphering the mechanism by which a plant "recognizes" and responds to NO could prove to be remarkably valuable toward the elucidation of the plant's global stress response and the engineering of tolerant crops.

Material and Methods

Plant material and growth conditions. This study was conducted using *M. truncatula* genotype Jemalong A17. After scarification, seeds were sown in sterile perlite:sand (3:1) pots and placed at 4°C for 4 d for stratification. Plants were grown in a growth chamber at 22/16°C day/night temperatures, at 60–70% RH, with a photosynthetic photon flux density of 100 μmol m^2s^{-1} and a 16/8 h photoperiod. Experiments were performed in triplicate using pooled samples (each "replicate" sample comprising tissues from a minimum of three independent plants).

SNP application treatment. Mature (40 d) and senescing (65 d) plants were vacuum-infiltrated with low (100 μM)and high (2.5 mM) concentrations of SNP and samples were obtained at 3 h (transient effect) and 24 h (long-term effect) as previously described.[61] Vacuum-infiltration was chosen as it represents the optimal method for SNP application.[62] Control samples were vacuum-infiltrated with dH_2O. **Supplemental Figure 1** represents a detailed outline of the experimental design followed. Leaf samples were flash frozen in liquid nitrogen and stored at −80°C for subsequent analyses.

Carotenoid and chlorophyll content. Leaf pigments were extracted from 9 mm-diameter leaf discs in dimethyl sulfoxide as described by Richardson et al.[63] Carotenoid and chlorophyll concentrations were determined using the equations described by Sims and Gamon.[64]

Lipid peroxidation assay. The extent of lipid peroxidation was determined from measurement of malondialdehyde (MDA) content resulting from the thiobarbituric acid (TBA) reaction as described by Hodges et al.,[65] using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Hydrogen peroxide and nitric oxide quantification. Hydrogen peroxide was quantified using the KI method, as described by Velikova et al.[66] NO content was measured using the Griess reagent in homogenates prepared with Na-acetate buffer (pH 3.6) as described by Zhou et al.[67] Nitrite-derived NO content was calculated by comparison to a standard curve of $NaNO_2$.

Nitrate reductase assay. The assay was performed essentially as described,[68] with some modifications. The buffer used for preparation of crude extracts contained 100 mM potassium phosphate (pH 7.5), 5 mM $(CH_3COO)_2Mg$, 10% (v/v) glycerol, 10% (w/v) polyvinylpyrollidone, 0.1% (v/v) Triton ×-100, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine (prepared fresh) and 1 mM 6-aminocaproic acid. Leaf tissue was extracted in the appropriate buffer using a mortar and pestle and the mixture was thoroughly homogenized. Cell extract was centrifuged at 14,000× g for 15 min and the clear supernatant was used immediately for measurement of enzyme activity. NR enzyme activity was expressed as specific enzyme activity (units/mg protein).

Histochemical detection of reactive oxygen species and lipid peroxidation. The detection of H_2O_2 in tissues was performed according to Thordal-Christensen et al.[69] with the following modifications. Detached leaves were incubated in 1 mg/ml 3,3'-diaminobenzidine (DAB)-HCl, pH 3.8 in the dark at room temperature for 8–10 h, then chlorophyll was removed by boiling in ethanol (96%, v/v) for 10 min. The assay was based on the instant polymerization of DAB (to form a reddish-brown complex which is stable in most solvents), as soon as it comes into contact with H_2O_2 in the presence of peroxidases. To determine the specificity of DAB staining, leaves were also stained in the presence of 200 units ml/1 catalase (bovine liver, Sigma-Aldrich) in a control experiment.

Leaf disks were stained with Schiff's reagent for stress-induced lipid peroxidation.[69,70] Leaf disks were stained with Schiff's reagent (Sigma) for 30 min, and rinsed with 0.5% (w/v) $K_2S_2O_5$ in 0.05 M HCl, which detects aldehydes originated from lipid peroxides.

RNA isolation, cDNA synthesis and real-time RT-PCR assay. Total RNA was prepared from leaves with the Qiagen RNeasy® Plant Mini Kit (Qiagen) followed by DNase digestion (RNase-free DNase Set; Qiagen). RNA integrity was analyzed spectrophotometrically and by gel electrophoresis. For real-time RT-PCR analyses, 1 μg of total RNA was converted into cDNA using Primescript 1st Strand Synthesis kit according to the manufacturer's protocol. Subsequently, real-time PCR was performed with Biorad IQ5 (Biorad). The reaction mix contained 4 μl cDNA in RT buffer (diluted 1:5), 0.75 μM of each primer (**Table S1**) and 1× master mix (SYBR Green Super Mix). Reactions were performed in triplicate and the thermocycler conditions were: 95°C for 5 min, then 40 cycles of 95°C for 30 sec, annealing temperature for 30 sec, 72°C for 30 sec, 80°C for 2 sec, plate read at 78°C, followed by 72°C for 10 min. The annealing temperature of the tested primers is 60°C and 53°C for the reference gene (**Table S1**). Relative quantification of gene expression and statistical analysis of all qRT-PCR data (pairwise fixed reallocation randomization test) were performed using the REST software according to Pfaffl et al.[71] Actin 11 gene was used as a housekeeping reference gene.[72]

Statistical analyses. Statistical analyses of all measurements (excluding qRT-PCR data) were performed using SPSS v.11 (SPSS Inc.,). Biochemical and physiological damage measurements were subjected to ANOVA, and then significant differences between individual means determined using Tukey's pairwise comparison test at the 5% confidence level.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplementary Material

Supplementary material may be found here:
www.landesbioscience.com/journals/psb/article/25479/

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